INVESTIGATIONS ON THE IN VITRO MORPHOGENETIC REACTION OF *MELISSA OFFICINALIS* L. SPECIES

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Keywords: lemon balm, micropropagation, morphogenetic reaction

Abstract: The paper presents some preliminary results concerning the in vitro initiation of *Melissa officinalis* species and the morphogenetic reaction of some explants on several hormonal formuli of the basal Murashige-Skoog medium. We recommended some medium formuli that are effective for this species' micropropagation as well as the appropriate accommodation to septic conditions of the neoplantlets obtained in vitro and for the regenerants' transfer to field.

INTRODUCTION

Melissa officinalis L. is a herbaceous, perennial plant of the *Lamiaceae* family, a native of the northern Mediterranean region. It is a xeromesophitic, moderate thermophile plant that is spread in sunny and also a bit shady places and is resistant to drought. It is sensitive to low temperatures, requiring mild winters. Its development is favourable on argillaceous earth and also on sandy, loamy ground. On vegetable soil its content of essential oils is lower. The aerial part of plant comprises 0.05 to 0.15% of volatile oil (that contains citronellal, citral, geraniol, linalool), polyphenols, tannins (3 to 6%), mucilages (12%), bitter substances etc. The seeds contain fat oil made up of linolenic, linoleic, oleic, palmitic and stearic acids (1-3, 5-8,10). The main action of its active principles, especially of volatile oil of *Melissa officinalis* is spasmolitic and sedative, recommended for gastro - intestinal spasms and cardiac neurosis. They are also known for an antiseptic, sedative, carminative, choleretic, mild laxative, stomachic, cicatrisant, galactagogue and insecticide, (2,3,6,8,10).

Considering this species economical importance we intended to find out some information considering its in vitro behaviour, the reaction of several explants on varied hormonal formuli, the possibility of identifying an effective micropropagation technology and the isolation of possible valuable genotypes. This kind of investigations were done by by others authors too, (4,7,9).

MATERIAL AND METHODS

The explant source for in vitro cultures initiation at *Melissa officinalis* were some individuals brought from Chalkidiki (Greece), grown in soil pots, in laboratory. We used shoot tips that were sterilized with a chloramine-T solution (5%) for 25 minutes, subsequently they were rinsed twice with sterile water and inoculated on hormone-free Murashige-Skoog (1962) medium or on MS supplemented with BAP (0.2-0.5 mg/l) or with kinetine (1 mg/l) and NAA (0.5 mg/l). The sterile neoplantlets obtained were used to test the morphogenetic reaction of different types of explants (nodes, shoot tips, internode, root and leaf fragments) on a series of hormonic formuli of MS medium. Cultures were grown in Erlenmayer fials of 100 ml (B type). Saccharose (25 g/l) was the charbon source and agar was used to solidify the nutritive medium. The inoculated fials were incubated in a half-climatised culture room within the 'Stejarul' Research Centre from Piatra Neamt (temperature was 23-25°C, light – about 2500 lux, continuous illumination). The neoplantlets provided on certain hormonal formuli were accommodated to septic conditions in a hydroponic system. The results are presented in table no.1 and figures 1-8.

RESULTS AND DISCUSSIONS

Our observations at the moment of in vitro culture initiation of *Melissa officinalis* species evinced the fact that including the plant in this culture system does not cause any particular problem and the use of chloramine -T (5% solution, for 25-30 minutes) to sterilize the explants proved to be effective. The use of basal hormone-free MS medium as well as MS supplemented with small amounts of BAP (0.2-0.5 mg/l) but especially with kinetine (1 mg/l) and NAA (0.5 mg/l) favoured the process of shoot development and generation of neoplantlets that are then

used to test the morphogenetic reaction of varied explants of lemon balm on different hormonal formuli of MS.

Nodes and shoot tips were most frequently used. When these explants were inoculated on basal MS, their reaction was neoplantlet generation, that generally have up to three basal shoots, rather long internodes (between 2 and 4 in about 3 weeks), well developed leaves and long roots with secondary branches. Nodes and shoot tips inoculated on MS supplemented with citokinines (BAP) provided neoplantlets characterized by vigorous multiple shoots of varied dimensions (fig. 1), quite thick stems and dark green leaves; sometimes a compact, small-sized callus is generated and it turns green at the contact surface between the explants and the culture medium. In other cases long roots with secondary branches appear between 2 successive transfers (about 30 days) or roots are provided only sporadically. The multiple shooting is enhaced on a culture medium containing kinetine (0.5 mg/l) and BAP (0.5 mg/l) but the growth rate is slower, root formation being also less intense.

In case of an MS medium supplemented with auxins the use of IAA (2 mg/l) and of nodes and shoot tips as explants favour either neoplantlet generation or just roots, the reaction of explants being a slow one. On a culture medium supplemented with NAA (2 mg/l) a friable, low proliferating cream callus appeared, other times it provided neoplantlets bearing shoots with long internodes and seldom long roots, but more frequently short and numerous roots growing from the node that contacts medium. Nodes and shoot tips placed on MS comprising IBA (2 mg/l) led to neoplantlet formation and they were more vigorous than on media containing IAA and NAA, the newly formed shoots having long internodes and the roots in a greater number, some of them more stronger and with secondary branches. The medium variant with 2 mg/l IBA seems to be appropriate to obtain vigorous neoplantlets in order to multiply some valuable genotypes of this species.

During our research a special attention was offered to nodes and shoot tips on media comprising combinations of auxins and citokinines (fig. 2, 3, 4), or citokinines and giberellins. It was ascertained that the best reaction of these explants was obtained on a medium supplemented with kinetine (1 mg/l) and NAA (0.5-1 mg) that favoured the generation of neoplantlets with a high speed growth, bearing strong stems and roots, sometimes much larger leaves, very long roots with many secondary branches (fig. 4). This hormonal formula is the most recommended for neoplantlet formation in order to micropropagate lemon balm. The MS variant containing BAP (1 mg/l) and IAA (0.5 mg/l) favoured the generation of neoplantlets with shoots and shorter internodes, smaller leaves sometimes arranged feather – like from the node, thinner stems, frail, long and whitish roots (fig. 2). In the case of an MS medium containing BAP (1 mg/l) and IBA (0.5 mg/l) the reaction of shoot tips and nodes was weaker, meaning that the newly generated neoplantlets were feeble and small-sized, bearing green-yellowish leaves, poor root formation was observed (as a rule secondary roots are fewer or even absent)(fig. 3). On an MS medium supplemented with BAP (1 mg/l) and NAA (0.5 mg/l) the neoplantlets were more vigorous than on the previous formula but less developed than on KN, IB and N; shoots were smaller-sized, leaves a bit yellowish, root formation less represented. Multiple shooting phenomenon was obviously stimulated on MS medium comprising BAP (1 mg/l) and giberellic acid (0.5 mg/l), shoots were small – sized, with reduced green-yellowish leaves and root formation did not occur. We ascertained that the reaction of explants and the strength of the biological material obtained on varied hormonal formuli clearly depended on the hormones within the nutritive medium and also on the vigour of the neoplantlets used as explant source.

We used some hormonic variants to test the reaction of other types of explants. We observed that the inoculation of internode fragments on MS supplemented with 2 mg/l 2,4-D (fig. 5) provided a compact, green-whitish callus with a medium proliferation speed, mostly formed at the ends of the fragments (conferring them a bar bells shape). If 2,4-D (0.5 mg/l) is combined with BAP (1 mg/l) internodes reacted more intensely generating a compact, green-whitish callus on the entire explant surface, especially at the ends where cell proliferation is much more intense. Root fragments had a similar reaction on this medium formula providing a cream-greenish callus (fig. 6), but its proliferation speed was lower than that of the internode fragments. Leaves reaction on the previous hormonic formuli was also similar to the mentioned one. On a medium comprising 2,4-D (2 mg/l) leaves generated a hard, small-dimension whitish callus in the petiole region. 2,4-D (0.5 mg/l) associated with BAP (1 mg/l) assured the proliferation of a compact, light green callus on the entire surface of the limb that contacts the nutritive medium (particularly in the petiole region), with a medium cell proliferation speed.

Within our research we also tried to test the reaction of the varied origin callus tissue on other hormonic formuli of MS medium. We observed, for instance, that by transfering the internode callus on media supplemented with 0.2-0.5 mg/l BAP it continued to proliferate slowly, turning cream and friable with an obvious tendency to degenerate (it turns brown on large areas). On certain regions it seldom provided short thick thorny roots. Internode callus transferred on MS medium supplemented with BAP and GA suffered the same reaction. Leaf callus on a medium comprising BAP (0.5 mg/l) had a better proliferation, maintained its consistency and colour, sporadically generating roots at the surface of the nutritive medium depending on the vigour of the neoplantlets used as explant source.

Caulogenesis was absent in both cases on the tested media formuli. The neoplantlets obtained on the varied media formuli were accommodated to septic environment in a hydroponic system (fig. 7). Due to their thin leaves plants frequently suffer water losses, this being the reason why their accommodation is not easy to accomplish, the room requiring a more humide atmosphere and a lower temperature (about 20°C) and major thermic changes must be avoided. The previously mentioned conditions permitted a more facile accommodation period and diminished losses of biological material.

During the spring of 2004 we transferred some douzins of in vitro obtained regenerants (fig. 8) on a plot belonging to ‘Stejarul’ Research Centre in Piatra Neamt. Generally those regenerants are quite frail, their leaves being very thin and that is why they stagnate for a long period of time after their transfer to field, but subsequently they resume growth processes. It is for a fact that the agro-meteorological conditions (lower temperatures and abundant rain) of the research region during the spring of 2004 were not the most appropriate ones for plant breeding. Nevertheless the regenerants developed well until September 2004 and certainly they will surpass the winter season without significant losses.

CONCLUSIONS

In vitro culture initiation of *Melissa officinalis* can be accomplished beginning with shoot tips drawn from field-grown or greenhouse plants, sterilised in a chloramine-T solution (5%) and inoculated on basal MS medium or on MS supplemented with kinetine and NAA.

The explants (nodes and shoot tips) provided neoplantlets on the majority of the hormonic formuli tested (comprising cytokinins, auxins or combinations of those two types of growth regulators). The best reaction was obtained on MS medium supplemented with kinetine and NAA, followed by IBA, NAA and even on hormone-free MS.

The addition of BAP and GA to the nutritive medium favoured multiple shooting and inhibited root formation of the new shoots that originate in nodes and shoot tips.

Internode, leaf and root fragments provided callus on media comprising 2,4-D. Callus formation process was more intense if 2,4-D was associated with BAP. The callus was compact, green in the case of internodes and leaves and cream for roots. Its proliferation from internode fragments was more intense.

The callus provided by internodes and leaves on MS supplemented with BAP or with BAP and GA maintained its characteristics, assured its proliferation and sporadically induced root formation on its surface. Caulogenesis was absent on the tested media formuli and generally the callus tended to degenerate on large areas.

In order to micropropagate *Melissa officinalis* we recommend the use of MS medium with kinetine and NAA, followed by those supplemented with IBA, NAA and even of basal MS. After a period of growth stagnation, field-grown regenerants resumed their growth, stems and leaves became more vigorous compared to their aspect during the accommodation period, with high survival chances the next unfavourable season.

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Tabel nr. 1 – The morphogenetic reaction of some lemon balm explants on varied hormonic formuli of the Murashige-Skoog medium

Var.	Explant	Medium formula	Growth regulators (mg/l)							Morphogenetic reaction and proliferation speed
			BAP	GA	IAA	IBA	NAA	KIN	2,4-D	
1	Nodes and shoot tips	A			2,0					Neoplantlets (+), roots (+); slow reaction
2	"	B	0,2-1,0							Neoplantlets with multiple shoots (++) and vigorous leaves, strong roots (+++) with secondary branches
3	"	BA	1,0		0,5					Neoplantlets with shorter internodes (++), leaves with a feather-like disposition, thin, long (++) whitish roots; multiple shooting (+) rarely
4	"	BB	0,5			0,5				Neoplantlets (+) with small shoots and yellowish leaves, root formation (+) less represented
5		BK	1,0					0,5		Neoplantlets (++) with multiple shoots (+++), shoots of different sizes, root formation (++) less intense
6		BG	1,0	0,5						Multiple shooting (+++), small shoots with small yellowish leaves, absence of root formation
7	"	BN	1,0				0,5			Neoplantlets (++) with 1 shoot per node, shoots of varied dimensions, leaves a bit yellow, root formation (++) less intense
8	"	IB				2,0				Well developped neoplantlets (+++) , long internodes, strong main root (+++) and numerous rather long secondary branches
9	"	N					2,0			Neoplantlets (++) with long shoots and internodes; roots (++) mostly short ones and surrounding the node, but also long roots with secondary branches; sometimes a thin basal layer of friable callus provided by nodes
10	"	KN					0,5	1,0		Neoplantlets (+++) with strong shoots and roots, internodes and thick roots; large dark green leaves
11	"	MS								Neoplantlets (+++) with vigorous shoots, long internodes, sometimes multiple shoots at basal node, strong root with secondary branches
12	Internode	BD	1,0						0,5	Compact callus (+++), green on the entire explant surface, mostly at the ends
14	Leaves	BD	1,0						0,5	Compact callus (++), light green colour, on the whole surface of the leaves in contact with the nutritive medium
16	"	D							2,0	Compact, whitish callus (+), generated particularly in the petiole region
17	Roots	BD	1,0						0,5	Compact callus (++), cream-greenish colour on entire explant surface
18	Internode callus	BG	1,0	0,5						Proliferative callus (++) that maintains its characteristics but does not differentiate
19	Leaf callus	B	0,5							Callus with a slow proliferation (++) sporadically providing thorn-like roots on its surface (+)

Fig.1. Neoplantlets generated on MS+0.2 mg/l BAP

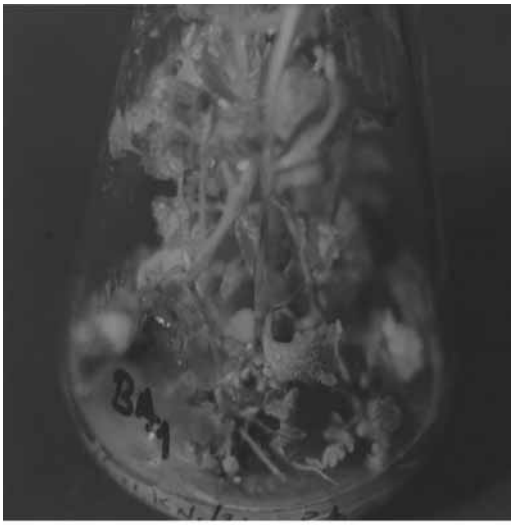

Fig. 2. Neoplantlets generated on MS+1 mg/l BAP+0.5 mg/l IAA

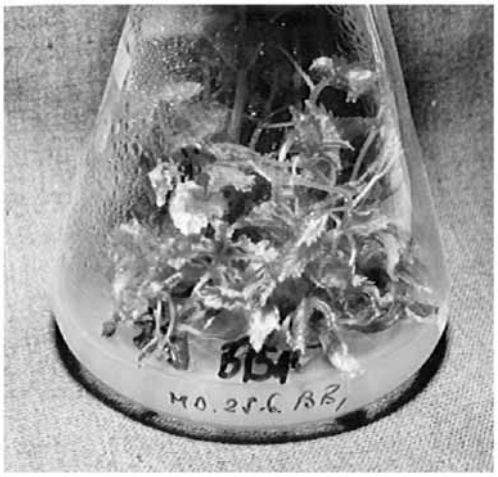

Fig.3. Neoplantlets generated on MS+0.5 mg/l BAP+0.5 mg/l IBA

Fig.4. . Neoplantlets provided on MS+1 mg/l kinetine+0.5 mg/l NAA

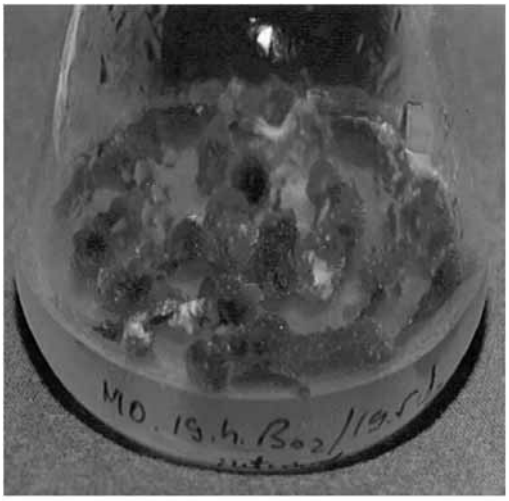

Fig.5. Callus from internodes on MS+2 mg/l 2.4-D

Fig.6. Callus originating from roots on MS+1 mg/l BAP+0.5 mg/l 2.4-D

Fig.7. Neoplantlet accommodation to septic environments (hydroponic system)

Fig.8. First year regenerants in field conditions